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Prevalence and identification of extended spectrum β-lactamases (ESBL) in *Escherichia coli* isolated from a tertiary care hospital in North-East India

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Extended-spectrum β-lactamases (ESBLs) are rapidly evolving group of β-lactamase enzymes produced by the Gram negative bacteria. In this study, we determined the antimicrobial sensitivity pattern of *Escherichia coli* isolates and prevalence of TEM, SHV and CTX-M genes in ESBL positive *E. coli* isolated from the patients admitted to a tertiary care hospital in North-East India. A total of 85 multidrug-resistant isolates of *E. coli* obtained from clinical samples; urine (n=80), sputum (n=3), body fluid (n=1), vaginal discharge (n=1) were screened for resistance to third generation cephalosporins. ESBL production in resistant isolates was determined by double disk synergy test (DDST) and phenotypic confirmatory test (PCT). ESBL positive isolates were subjected to PCR for detection of TEM, SHV and CTX-M genes. Imipenem was found to be most effective against *E. coli* (susceptible isolates 96.47%) while ciprofloxacin was the least effective antibiotic (resistant isolates 60%). Among 33 ESBL positive isolates confirmed via PCT, preponderance in female population (60.6%) was noted. The most prevalent gene was bla_{SHV} (63.04%) followed by bla_{TEM} and $bla_{CTX\text{-}M}$ (60.86 and 54.34%, respectively) in ESBL positive *E. coli*. Most of the extensively used antibiotics, appear to be ineffective against the ever-mutating bacteria. This resistance urges cautious antimicrobial management on priority. Further, it helps in effectively designing the chemotherapeutic regimen for patients of a particular geographic area.

Keywords: CTX-M, Drug resistance, MDR, Public health, SHV, TEM.

Drug resistance in microbes is a growing major public health issue. Although the epidemiology of resistance can exhibit a remarkable geographical variability, it is a strong concern for the medical community due to its global dimension and alarming magnitude[1]. Since their first description >30 years ago, pathogens producing extended-spectrum beta lactamases (ESBLs) have become an increasing cause of clinical concern[2]. They have been detected in *Klebsiella* sp., and later in *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* and other gram-negative bacilli[3], and also gram positive *Staphylococcus aureus*[4]. These enzymes act by hydrolyzing oxyimino-cephalosporins conferring resistance to cefotaxime, ceftazidime and ceftriaxone i.e., third generation cephalosporins as well as to monobactams, such as aztreonam. Increased resistance to widely used antibiotic cefotaxime in late 1980's has led to characterization of CTX-M and SHV genes as responsible factors imparting resistance to wider range of penicillins and third-generation cephalosporins[5]. ESBL producing gram negative rods represent a momentous challenge to the antibiotic armamentarium worldwide[6], particularly in the form of hospital-based outbreaks of ESBL-producing organisms, especially *Escherichia coli* producing TEM, SHV or CTX-M-type enzymes[6,7]. Determination of TEM, SHV and CTX-M genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can bring in useful data about their epidemiology and risk factors associated with these infections.

Here, we studied the prevalence of mainly TEM, SHV and CTX-M genes responsible for ESBL production amongst the ESBL positive *E. coli* isolated from various samples (urine, blood, sputum, wound, abscesses, catheter, peritoneum and cerebrospinal

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fluid) from patients hospitalized in different units of Gauhati Medical College and Hospital, Gauhati.

Materials and Methods

Bacterial isolates

A total of 85 consecutive non-duplicate clinical isolates of multidrug resistant *E. coli* [Urine (n=80), sputum (n=3), body fluid (n=1) and vaginal discharge (n=1)] received in the Genetic Laboratory, Dept. of Microbiology, Gauhati Medical College and Hospital (GMCH), Assam, during July-December 2011 were included in this study.

Antimicrobial susceptibility testing

Isolates were tested by the disk diffusion method on Mueller Hinton agar (Hi-Media, Mumbai) following the zone size criteria recommended by ITS CLSI, 2011. The antibiotics tested (in µg) were ampicillin-cefpodoxime (10), aztreonam (30), cefepime (30), cefotaxime (30), ticarcillin-clavulanate (75/10), imipenem (10), cefoxitin (30), ceftazidime (30), amoxicillin-clavulanate (20/10), piperacillin-tazobactam (100/10), ticarcillin (75), piperacillin (100), ceftriaxone (30), cotrimoxazole (25), ciprofloxacin (5), amikacin (30), tobramycin (10), doxycyclin (30), meropenem (10), netilmicin (30), cefoperazone (75), cefuroxime (30) and ceftazidime-clavulanic acid (30/10).

ESBL screening and confirmation by phenotypic methods

The isolates showing resistance to one or more third-generation cephalosporins were tested for ESBL production by double disk diffusion test (DDDT) using four discs, *viz*., cefotaxime (CTX; 30 µg) cefotaxime + clavulanic acid (10 µg), cefatzidime (CAZ; 30 mg) and ceftazidime+clavulanic acid (10 mg). The inoculum and incubation conditions were the same as for standard disk diffusion recommendations. A >5 mm increase in zone diameter was designated as ESBL positive when tested alone or in combination with clavulanic acid. *Klebsiella pneumoniae* ATCC700603 was used as a positive control and *E. coli* ATCC25922 was used as negative control.

Detection of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes

Plasmid DNA was extracted from all the screening positive isolates using the plasmid isolation kit provided by the Qiagen (Genetix Biotech Asia Ptv. Ltd., India) as per the manufacturer's protocol. Detection of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes from total bacterial DNA was performed with a subset of primers that amplified 717 bp $bla_{TEM,}$ 930 bp bla_{SHV} and 554 bp bla_{CTX-M}, coding region *Escherichia* sp. genome. Primers (provided by Qiagen, India) used are listed in Table 1.

Table 1—Primers (Forward and reverse) used for the amplification of β-lactamase resistance genes

Gene	Primer Sequence
blaTEM[7]	F: 5′CTTCCTGTTTTTGCTCACCCA-3′ R: 5′TACGATACGGGAGGGCTTAC-3′
blaSHV[8]	F: 5′GGGTTATTCTTATTTGTCGC-3′ R: 5′TTAGCGTTGCCAGTGCTC-3′
blaCTX-M[9]	F: 5′SCSATGTGCAGYACCAGTAA-3′ R: 5′CCGCRATATGRTTGGTGGTG-3′

Reactions were performed in a DNA thermal cycler (Thermo Scientific, India) in 20 µl mixtures containing 15 U Taq polymerase (Qiagen, India), 1X buffer consisting of 10 mM Tris HCL (pH-8.3), 1.5 mM $Mgcl_2$, 50 mM KCL, 0.01 µg of gelatin, and each deoxynucleoside triphosphate @50 pmol/µL.

PCR profiles for bla $_{TEM}$; *bla* $_{SHV}$; and *bla* $_{CTXM}$—It was an initial denaturation at 95°C for 5 min followed by denaturation at 95°C for one min, annealing at 50°C for bla_{TEM}, 54°C for bla_{SHV}; and 58°C for bla_{CTXM} for one min and extension at 72°C for one min and a final extension cycle at 72°C for 10 min. Denaturation, annealing and extension was repeated for 30 cycles before the final extension step.

PCR products were analysed by electrophoresis with 1.5% agarose gel. After staining with ethidium bromide, the gel was photographed on an UV trans-illuminator by gel documentation system (Perkin Elmer).

Results

Out of the 85 samples (Male 40 and Female 45) collected for the study, maximum number (n=19) were from the age group of 21-30 and 41-50. The samples collected were mostly from urine (94.11%). Other sources of clinical sample were body fluid (1.17), sputum (3.52) and vaginal discharge (1.17%).

The Disk diffusion method according to Clinical and Laboratory Methods Institute (CLSI), 2011 guidelines showed 46 samples suspected as ESBL positive, whereas the confirmatory tests such as Double disk synergy test (DDST) and Phenotypic Confirmatory Test (PCT) identified 30.58% (26/85) and 38.82% (33/85) as ESBL producing strains (Table 2). Out of 33 ESBL positive isolates, 60.6% were from female patients and the age group 31-40 had the maximum number of ESBL producers i.e., 10 (7 females and 3 males). ESBL prevalence from the major clinical specimen viz. Urine was found to be 38.75% (31/80).

In vitro antibiotic susceptibility test of *E. coli* isolates revealed that majority of them were susceptible to imipenem (96.47%), followed by

piperacillin-tazobactam and netilmicin (90.58%), amikacin (88.23%), tobramycin (78.82%), etc. (Table 3). Higher resistance rates (>50%) were seen against ciprofloxacin (60%), doxacyclin (58.82%), cefpodoxime (56.47%), ticarcilin (55.29%), cefoperazone and cotrimoxazole (51.76%).

Table 2— Age- and Sex- wise distribution of ESBL positive isolates among patients.

Age Group (years)	No. of clinical samples	No. of ESBL positive isolates		Total	(%)
		Male	Female		
1-10	2	1	0	1	3.03
11-20	5	1	2	3	9.09
21-30	19	2	3	5	15.15
31-40	16	3	7	10	30.30
41-50	19	2	5	7	21.21
51-60	12	3	1	4	12.12
61-70	5	0	1	1	3.03
71-80	7	1	1	2	6.02
Total	85	13	20	33	

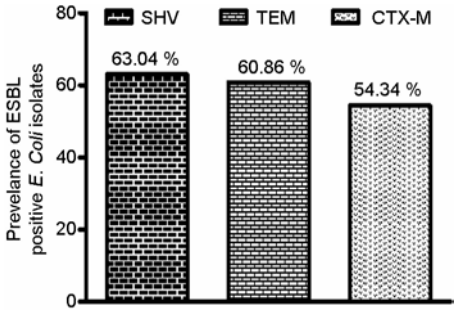

Fig. 1—Prevalence (in terms of percentage) of different ESBL genes among ESBL positive *E. coli* isolates.

Plasmid DNA isolated from 33 ESBL producing *E. coli* isolates were subjected to PCR with the TEM, SHV and CTX-M specific primers. The prevalence of bla_{SHV} among tested isolates was 63.04%, while that of bla_{TEM} and bla_{CTX-M} was found to be 60.86 and 54.34%, respectively (Fig. 1). The agarose gel electrophoresis displayed the presence of different ESBL genes in the amplified samples as shown in Fig. 2 and their sizes were found to be 514, 717 and 930 bp, respectively for bla_{CTX-M}. bla_{TEM} and bla_{SHV} genes.

Table 3—Antibiogram showing sensitivity pattern of *E. coli* isolates to various antibiotic disks during *in vitro* testing. Total number of isolates tested 85.

Antibiotic	No. of isolates		Percentage of	
	Sensitive	Resistant	Sensitivity	Resistance
Imipenem (IPM)	82	3	96.47	03.52
Piperacillin-Tazobactum (PIT)	77	8	90.58	09.41
Netilmicin (NIT)	77	8	90.58	09.41
Amikacin (AK)	75	10	88.23	11.76
Tobramycin (TOB)	67	18	78.82	21.17
Cefoxitin (CX)	55	30	64.70	35.29
Ceftazidime (CAZ)	53	32	62.35	37.64
Meropenem (MRP)	53	32	62.35	37.64
Amoxycillin-Clavulanic acid (AMC)	53	32	62.35	37.64
Cefepime (CPM)	51	34	60.00	40.00
Aztreonam (AZT)	49	36	57.64	42.35
Cefotaxime (CTX)	46	39	54.11	45.88
Ticarcillin-Clavulanic acid (TCC)	46	39	54.11	45.88
Piperacillin (PI)	45	40	52.94	47.05
Ceftriaxone (CTR)	44	41	51.76	48.23
Cefuroxime (CXM)	43	42	50.58	49.41
Cefoperazone (CS)	41	44	48.23	51.76
Cotrimoxazole (COT)	41	44	48.23	51.76
Ticarcillin (TI)	38	47	44.70	55.29
Cefpodoxime (CPD)	37	48	43.52	56.47
Doxycyclin (DOX)	35	50	41.17	58.82
Ciprofloxacin (CIP)	34	51	40.00	60.00

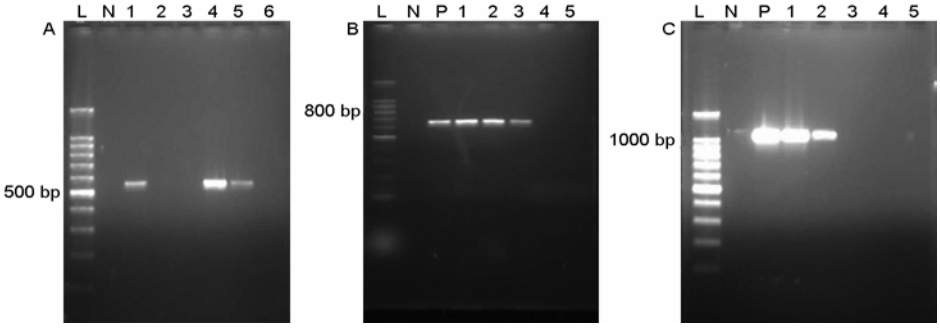

Fig. 2—Agarose gel showing (A) 554 bp fragments of bla_{CTX-M} gene; (B) 717 bp fragments of bla_{TEM} gene; and (C) 930 bp fragments of bla_{SHV} gene. [Lanes 1, 4 and 5 in (A); 1, 2 and 3 in (B); and 1 and 2 in (C) were loaded with ESBL positive isolates while lanes 2, 3 and 6 in (A); 4 and 5 in (B); and 3, 4 and 5 in (C) were loaded with ESBL negative isolates. Lane 'N' had negative control - *E. coli* ATCC 25922; Lane 'P', positive control - *E. coli* ATCC 35218 in (B); and *K. pneumoniae* ATCC 700603 in (C); Lane 'L' was loaded with 1.5 kbp ladder].

Discussion

The extensive use of extended spectrum β-lactam agents *viz.*, third-generation cephalosporins and monobactams in the late 1970's led to the emergence of resistant strains, named as ESBLs[10]. Resistance conferred by ESBLs extended from third and fourth generation cephalosporins to aztreonams and extended-spectrum penicillins[11]. ESBL producing organisms have become common in hospitals and nursing homes including ICU. In a study on hospital inmates for presence of ESBLs, nearly three-fourth of the patients with ESBL producing strains didn't have prior history in recent three months[12]. India, being a heavily populated country with poor sanitation and drinking water problems, represents largest reservoir of CTX-M ESBL genes alongside China[13].

E. coli is a major cause of urinary tract infections (UTI) worldwide, representing the foremost organism isolated from urine specimens[14]. Urine represents a major (94.11%) clinical specimen for the *E. coli* isolates in this study, similar to Nasehi *et al.* [15]. More than 60% of the clinical isolates were from patients in the age group of 21-50, indicating it as the most common age group for development of UTI. Mathai *et al.*[16] reported ESBL-producing *Enterobacteriaceae* going epidemic in India with 62-100% of *E. coli* and *Klebsiella* strains . These extended spectrum β-lactamases are susceptible to carbepenems, cephamycin, temocillin and ceftibutin (ineffective against few SHV derivatives)[17]. In the present study, imipenem was found to be most effective against majority of *E. coli* isolates where 96.47% of the specimens were vulnerable to it, followed by piperacillin-tazobactam and netilmicin (90.58%) and amikacin (88.23%).

A Surveillance of antimicrobial resistance in India SARI study by Mahoharan *et al.*[18] and prevalence study at a tertiary care hospital by Dalela[19] showed comparable results where carbepenem was found to be most effective and amikacin and piperacillin-tazobactam were showing more than 80% effectiveness. Based on retrospective and current studies, carbapenems are looking most potent against the menace of ESBL producing Gram negative bacilli. Carbepenems, such as imipenem, ertapenem and meropenem resists ESBL-producing strains more successfully than other β-lactam antibiotics, with ESBL positive isolates showing 100% susceptibility against imipenem[20].

When employing CLSI guidelines for testing ESBL producing isolates, a few bacteria may yield false positive susceptibility results for certain extended-spectrum cephalosporins[21]. Double disk synergy test is preferred for its easy interpretation[22] and sensitivity range 79-96% that can be improved by reducing interdisk width to 20 mm[23]. Phenotypic confirmatory tests (PCT) have more sensitivity and specificity compared to their genotypic counterparts, except the instances where, ESBL negative *enterobacteriaceae*, synthesize SHV-1 in excess giving false positive results [3]. In accordance with CLSI, 2011 guidelines, organisms showing positive PCT should be reported as resistant to cephalosporins (cephamycins, cephoxitin and cephotetan being the exception) and aztreonam, irrespective of the MIC of cephalosporins[24].

In this study, out of 85 clinical isolates of *E. coli*, 46 were found to be ESBL producers via the CLSI method of ESBL detection, whereas the DDST confirmed only 26 (30.58%) isolates as positive ESBL producers, and the PCT detected 33 (38.82%) samples as ESBL positive. In a related study by Shoorashetty *et al.*[25], PCT detected 41% isolates as ESBL positive of the total 200 specimens, corroborating with our findings. DDST and PCT done by Metri *et al.*[26] on 126 *E. coli* isolates detected 28.6 and 31.7% as ESBL producers, respectively. ESBL prevalence in Pakistan and Iran have been reported to be higher than India[27,28]. MYSTIC program data from Europe during the period of 1997-2000 showed significant variation in prevalence of ESBL throughout the continent. ESBL occurrence in Russia, Poland and Turkey was higher, ranging 39-47% while that of Germany was found to be as low as 1.5%[29], matching up with the Scandinavian prevalence percentage of <1%[30]. Prevalence of 30-60% was found for the South American countries of Brazil, Columbia and Venenzuela[31-33], while it is interestingly lower in adjacent North America where the average incidence in US is reportedly only 3%[34]. In Asia, study conducted in Japan showed lower prevalence (5%) of ESBL menace whereas it was reported higher in Thailand, China and Australia in the next decade indicating a rise in ESBL producing organisms over time[3,35,36].

Ciprofloxacin has been the drug of choice for treatment of UTI[11]. Last decade has seen the rise of flouroquinolone resistant and multi-drug resistant *E. coli* isolates in various geographical regions[37]. In the present study, resistance to ciprofloxacin was seen among 60% of the bacterial isolates. A comprehensive North American (NAUTICA) study reported a much

lower incidence of resistance against ciprofloxacin (5.5%) among *E. coli* isolates[38]. This study also noticed higher resistance rates (increased number of antibiotic resistant isolates) in USA than in Canada[38]. Studies done in Gaza Strip and Turkey reported lower resistance rate (12%) of *E. coli* isolates for ciprofloxacin[39,40]. Resistance rates in *E. coli* isolates from women in Hong Kong were found to be comparatively higher at 22.1%, which possibly explains the higher frequency of UTI in women[41,42]. Results from SMART program 2009-2010 revealed that ciprofloxacin was not effective against ESBL positive isolates, whereby a much higher worldwide resistance rate of 85.4% to ciprofloxacin was noted[43]. This study justifies the higher rate observed in our study.

ESBL are the result of point mutations in the TEM-1 and TEM-2 and SHV-1 genes[44]. >130 TEM-type and >50 SHV-type β-lactamases have been reported from various places before the start of 21st century. A newer type, called CTX-M, was also being reported, having greater activity against cefotaxime than ceftazidime[34,45]. Isolates from India also harbour this newer CTX-M form in addition to the TEM- and SHV- type enzymes[13,16]. In the present study, bla_{SHV} gene was most prevalent (63.04%) among *E. coli* isolates followed by bla_{CTX-M} (60.86%) and bla_{TEM} (54.34%). All the three ESBL types were more or less equally prevalent in the bacterial strains, with majority of the isolates possessing more than one gene type. Goyal *et al*. found bla_{CTX-M} as the most common gene with a prevalence rate of 85.4% at a hospital in Lucknow, India[46]. In Riyadh, Saudi Arabia, the prevalence of SHV gene in ESBL isolates in *K. pneumoniae* has been reported to be as high as 97.3%[47]. A study in Chennai, India observed higher incidence of bla_{CTX-M} (50%) than bla_{SHV} (14%) in *E. coli* isolates[48]. A study conducted in tertiary care hospitals in central India reported high prevalence of CTX-M and low prevalence of SHV gene among ESBL positive isolates from hospital waste water[49]. CTX-M-15 is most frequent subtype of CTX-M found in India; a study published in 2006 reported CTX-M-15 as the sole CTX-M subtype among *E. coli* and *K. Pneumoniae* isolates[50].

This study showed preponderance of ESBL positive isolates in female patients. Out of 33 ESBL positive isolates, 60.6% (20/33) of the samples were from female population. Prior studies Ho *et al*.[41], have also reported greater proportion of female patients possessing ESBL positive *entero-bacteriaceae*. Most of the ESBL positive *E. coli*, 30.30%, was detected in the age group of 31-40 followed by 41-50 age group (21.21%).

In this study, we analyzed *E. coli* strains for the presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. It is likely that other ESBL genes (bla_{OXA}, bla_{PER}), though uncommon, may be contributed to the observed resistance pattern. Overall, the present study provides the antibiotic resistance pattern of organism in this geographic area. Further, it helps in defining proper antibiotic usage and therapy selection by clinicians, and thereby prevent the emergence of antibiotic resistance among the pathogens against the present effective antibiotics. In the recent years, carbapenemase such as NDM-1 has been detected in conjunction with ESBLs in the *E. coli* and *K. pneumoniae* strains in different parts of the world[51-53]. This is a cause for much concern as these carbapenemases work against carbapenems, for which the ESBL producing organisms are most susceptible. Though recent works such as Kainthola *et al*.[4] who have demonstrated electric stimulations to effectively check the growth of community acquired *Staphylococcus aureus* (CA-MRSA) and reversal of β-lactam resistance, the pathogens will likely overcome all the presently marketed antibiotics. In order to prevent such a situation, we need to implement a well-judged use of effective antibiotics.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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